

ANTISENSE MODULATION OF TRADD EXPRESSION**INTRODUCTION**

This application is a continuation of U.S. Serial No. 09/763,748 filed May 29, 2001, which is the U.S. National
5 Phase of PCT/US99/19614 filed August 25, 1999, which is a continuation of 09/143,212 filed August 28, 1998, now issued as U.S. Patent No. 6,077,672, each of which are herein incorporated by reference in their entireties.

FIELD OF THE INVENTION

10 The present invention provides compositions and methods for modulating the expression of TRADD. In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human TRADD. Such
15 oligonucleotides have been shown to modulate the expression of TRADD.

BACKGROUND OF THE INVENTION

One of the principal mechanisms by which cellular regulation is effected is through the transduction of
20 extracellular signals into intracellular signals that in turn modulate biochemical pathways. Examples of such extracellular signaling molecules include growth factors, cytokines, and chemokines. The cell surface receptors of these molecules and their associated signal transduction
25 pathways are therefore one of the principal means by which cellular behavior is regulated. Because cellular phenotypes are largely influenced by the activity of these pathways, it is currently believed that a number of disease states and/or disorders are a result of either aberrant

activation or functional mutations in the molecular components of signal transduction pathways. Consequently, considerable attention has been devoted to the characterization of these proteins.

5 For example, the polypeptide cytokine tumor necrosis factor (TNF) is normally produced during infection, injury, or invasion and serves as a pivotal mediator of the inflammatory response. In recent years, a number of in vivo animal and human studies have demonstrated that
10 overexpression of TNF by the host in response to disease and infection is itself responsible for the pathological consequences associated with the underlying disease. For example, septic shock as a result of massive bacterial infection has been attributed to infection-induced
15 expression of TNF. Thus, systemic exposure to TNF at levels comparable to those following massive bacterial infection has been shown to result in a spectrum of symptoms (shock, tissue injury, capillary leakage, hypoxia, pulmonary edema, multiple organ failure, and high mortality rate) that is virtually indistinguishable from septic shock syndrome (Tracey and Cerami, *Ann. Rev. Med.*, **1994**, 45, 491-503). Further evidence has been provided in animal models of septic shock, in which it has been demonstrated that systemic exposure to anti-TNF neutralizing antibodies block
25 bacterial-induced sepsis (Tracey and Cerami, *Ann. Rev. Med.*, **1994**, 45, 491-503). In addition to these acute effects, chronic exposure to low-dose TNF results in a syndrome of cachexia marked by anorexia, weight loss, dehydration, and depletion of whole-body protein and lipid.
30 Chronic production of TNF has been implicated in a number of diseases including AIDS and cancer (Tracey and Cerami, *Ann. Rev. Med.*, **1994**, 45, 491-503).

 Studies examining the molecular events associated with TNF exposure have revealed that activation of the
35 transcription factor NF-kappa-B is a critical component of

many of the effects attributed to TNF (Tewari and Dixit, *Curr. Opin. Genet. Dev.*, **1996**, 6, 39-44). More recently, the intracellular protein TRADD has been identified as a critical link between TNF receptor binding and downstream
5 activation of NF-kappa-B. Thus, overexpression of native TRADD was shown to activate NF-kappa-B in the absence of TNF and dominant negative mutants of TRADD have been shown to block TNF-induced NF-kappa-B activation (Tewari and
10 Dixit, *Curr. Opin. Genet. Dev.*, **1996**, 6, 39-44). A second effect of TNF in many cell types is the induction of apoptosis, or programmed cell death. As with NF-kappa-B activation, TRADD overexpression has been shown to mimic
TNF induction of apoptosis as well (Darnay and Aggarwal, *J. Leukoc. Biol.*, **1997**, 61, 559-566; Tewari and Dixit, *Curr.*
15 *Opin. Genet. Dev.*, **1996**, 6, 39-44).

As a result of these advances in the understanding of TNF overexpression in certain disease states, there is a great desire to provide compositions of matter which can inhibit the cellular effects elicited by TNF. In vitro
20 studies have shown that maximal cellular responses to TNF are elicited when as little as 10% of TNF cell membrane receptors are occupied (Tracey and Cerami, *Ann. Rev. Med.*, **1994**, 45, 491-503). These data indicate that downstream effector proteins such as TRADD are the rate-limiting step
25 of TNF action and would therefore serve as the most efficient targets for inhibition of TNF-induced events.

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of TRADD. Antisense oligonucleotides capable of inhibiting TRADD function may
30 therefore prove to be uniquely useful in a number of therapeutic, diagnostic and research applications.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding TRADD, and which
5 modulate the expression of TRADD. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of TRADD in cells or tissues comprising contacting said cells or tissues with one or
10 more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of TRADD by administering a therapeutically or
15 prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in
20 modulating the function of nucleic acid molecules encoding TRADD, ultimately modulating the amount of TRADD produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding TRADD. As used herein, the terms "target nucleic
25 acid" and "nucleic acid encoding TRADD" encompass DNA encoding TRADD, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal
30 function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be

interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of TRADD. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding TRADD. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A

minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and
5 "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative
10 start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons
15 that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding TRADD, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one
20 of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to
25 about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous
30 nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation
35 termination codon, is also a region which may be targeted

effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including
5 nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus
10 including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an
15 mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions,
20 known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred
25 target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It
30 has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently
35 complementary to the target, i.e., hybridize sufficiently

well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick,

5 Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for
10 precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to
15 each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable"
20 and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense
25 compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or
30 RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo

assays or therapeutic treatment, or in the case of in vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense
5 oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a
10 biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as
15 therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic
20 modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of
25 ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions
30 which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of
35 nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the

backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

5 Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates,
10 phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and
15 those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the
20 preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;
25 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; and 5,697,248, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do
30 not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside
35 linkages. These include those having morpholino linkages

(formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene
5 containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the
10 preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;
15 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the
20 sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has
25 been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are
30 retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of
35 which is herein incorporated by reference. Further

teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and
5 oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$ [known as a methylene (methylimino) or MMI backbone], $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$ and $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$ [wherein the native phosphodiester backbone is represented as $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$
10] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

15 Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-, S-, or N-alkenyl, O, S- or N-alkynyl, or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1
20 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{OCH}_3$, $\text{O}(\text{CH}_2)_n\text{NH}_2$, $\text{O}(\text{CH}_2)_n\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{ONH}_2$, and $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides
25 comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3 , OCN, Cl, Br, CN, CF_3 , OCF_3 , SOCH_3 , SO_2CH_3 , ONO_2 , NO_2 , N_3 , NH_2 , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino,
30 substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred
35 modification includes 2'-methoxyethoxy ($2'-\text{O}-\text{CH}_2\text{CH}_2\text{OCH}_3$, also

known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also
5 known as 2'-DMAOE, as described in United States patent application Serial Number 09/016,520, filed on January 30, 1998, which is commonly owned with the instant application and the contents of which are herein incorporated by reference.

10 Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in
15 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars
20 structures include, but are not limited to, U.S.:
4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;
5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;
5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531
5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,700,920; and
25 5,858,221.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and
30 guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of
35 adenine and guanine, 2-propyl and other alkyl derivatives

of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as

well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

5 Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but
10 are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1994**, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*,
15 **1992**, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1993**, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, 259, 327-330; Svinarchuk et al.,
20 *Biochimie*, **1993**, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, 18, 3777-3783), a polyamine or a
25 polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*,
30 **1995**, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, 277, 923-937.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but

are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105;
5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717,
5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802;
5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;
5 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;
4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582;
4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;
5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;
5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,
10 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;
5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and
5,688,941, certain of which are commonly owned with the
instant application, and each of which is herein
15 incorporated by reference.

It is not necessary for all positions in a given
compound to be uniformly modified, and in fact more than
one of the aforementioned modifications may be incorporated
in a single compound or even at a single nucleoside within
20 an oligonucleotide. The present invention also includes
antisense compounds which are chimeric compounds.
"Chimeric" antisense compounds or "chimeras," in the
context of this invention, are antisense compounds,
particularly oligonucleotides, which contain two or more
25 chemically distinct regions, each made up of at least one
monomer unit, i.e., a nucleotide in the case of an
oligonucleotide compound. These oligonucleotides typically
contain at least one region wherein the oligonucleotide is
modified so as to confer upon the oligonucleotide increased
30 resistance to nuclease degradation, increased cellular
uptake, and/or increased binding affinity for the target
nucleic acid. An additional region of the oligonucleotide
may serve as a substrate for enzymes capable of cleaving
RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is
35 a cellular endonuclease which cleaves the RNA strand of an

RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be
5 obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary,
10 associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides,
15 oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.:
20 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through
25 the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well
30 known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector
35 constructs designed to direct the in vivo synthesis of

antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor
5 targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not
10 limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295;
15 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of
20 such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and
25 pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an
30 active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to
35 the methods disclosed in WO 93/24510 to Gosselin et al.,

published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of
5 the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline
10 earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine,
15 N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt
20 in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility
25 in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of
30 the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and
35 include basic salts of a variety of inorganic and organic

acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid,

hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of TRADD is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding TRADD, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding TRADD can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such

detection means for detecting the level of TRADD in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers,

diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions and/or formulations
5 comprising the oligonucleotides of the present invention may also include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts,
10 chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). One or more penetration enhancers from one or more of these broad
15 categories may be included. Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate,
20 monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arichidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate,
25 myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7:1, 1-33; El-Hariri et al., *J. Pharm. Pharmacol.*, **1992**, 44, 651-654).
30 Examples of some presently preferred fatty acids are sodium caprate and sodium laurate, used singly or in combination at concentrations of 0.5 to 5%.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and

fat-soluble vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their
5 synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. A presently preferred bile salt is chenodeoxycholic acid (CDCA) (Sigma Chemical Company, St.
10 Louis, MO), generally used at concentrations of 0.5 to 2%.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations. Preferred combinations include CDCA
15 combined with sodium caprate or sodium laurate (generally 0.5 to 5%).

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate
20 and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 92-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7:1, 1-
25 33; Buur et al., *J. Control Rel.*, **1990**, 14, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and
30 polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 92-191); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, **1988**, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 92-191); and non-steroidal
5 anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, **1987**, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (i.e., does
10 not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation.
15 The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition
20 between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated oligonucleotide in hepatic tissue is reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-
25 isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, **1995**, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, **1996**, 6, 177-183).

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a
30 pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration

in mind so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents Nos. 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the antisense compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the in vivo stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, **1995**, 6, 698-708).

Certain embodiments of the invention provide for liposomes and other compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 1206-1228). Antiinflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in

compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Examples of antisense oligonucleotides include, but are not limited to, those directed to the following targets as disclosed in the indicated U.S. Patents, or pending U.S. applications, which are commonly owned with the instant application and are hereby incorporated by reference, or the indicated published PCT applications: raf (WO 96/39415, WO 95/32987 and U.S. Patent Nos. 5,563,255, issued October 8, 1996, and 5,656,612, issued August 12, 1997), the p120 nucleolar antigen (WO 93/17125 and U.S. Patent No. 5,656,743, issued August 12, 1997), protein kinase C (WO 95/02069, WO 95/03833 and WO 93/19203), multidrug resistance-associated protein (WO 95/10938 and U.S. Patent No. 5,510,239, issued March 23, 1996), subunits of transcription factor AP-1 (pending application U.S. Serial No. 08/837,201, filed April 14, 1997), Jun kinases (pending application U.S. Serial No. 08/910,629, filed August 13, 1997), MDR-1 (multidrug resistance glycoprotein; pending application U.S. Serial No. 08/731,199, filed September 30, 1997), HIV (U.S. Patent Nos. 5,166,195, issued November 24, 1992 and 5,591,600, issued January 7, 1997), herpesvirus (U.S. Patent No. 5,248,670, issued September 28, 1993 and U.S. Patent No. 5,514,577, issued May 7, 1996), cytomegalovirus (U.S. Patents 5,442,049, issued August 15, 1995 and 5,591,720, issued January 7, 1997), papillomavirus (U.S.

Patent 5,457,189, issued October 10, 1995), intercellular adhesion molecule-1 (ICAM-1) (U.S. Patent 5,514,788, issued May 7, 1996), 5-lipoxygenase (U.S. Patent 5,530,114, issued June 25, 1996) and influenzavirus (U.S. Patent 5,580,767, issued December 3, 1996). Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to

illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

5 Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling
10 VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the
15 wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids*
20 *Research*, **1993**, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

25 2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was
30 synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro

atom is introduced by a S_N2 -displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of
5 the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

10 The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group
15 was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups.
20 Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure
25 in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

2'-Fluorodeoxycytidine

30 2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-

fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column,

packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total
5 yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a
10 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the
15 reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The
20 combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was
25 recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set
30 aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to

-5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was
5 added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble
10 solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-
15 **methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was
20 evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The
25 vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and
5 benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted
10 with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were
15 evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L).
20 Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction
25 mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column
30 using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-(Aminooxyethyl) nucleoside amidites and 2'-(dimethylaminooxyethyl) nucleoside amidites

Aminooxyethyl and dimethylaminooxyethyl amidites are prepared as per the methods of United States patent applications serial number 10/037,143, filed February 14, 1998, and serial number 09/016,520, filed January 30, 1998, each of which is commonly owned with the instant application and is herein incorporated by reference.

Example 2

10 Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

15 Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. 25 Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference. 30

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared
5 as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein
10 incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as
15 described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also
20 identified as MMI linked oligonucleosides, methylenedi-methylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligo-
25 nucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are
30 herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

5 PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*,
10 **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

15 Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked
20 nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are
25 also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric

Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligo-
30 nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above.

Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to ½ volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometer.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

**[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy
Phosphorothioate]--[2'-O-(2-Methoxyethyl)
Phosphodiester] Chimeric Oligonucleotides**

[2'-O-(2-methoxyethyl phosphodiester)--[2'-deoxy
5 phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester]
chimeric oligonucleotides are prepared as per the above
procedure for the 2'-O-methyl chimeric oligonucleotide with
the substitution of 2'-O-(methoxyethyl) amidites for the
2'-O-methyl amidites, oxidization with iodine to generate
10 the phosphodiester internucleotide linkages within the
wing portions of the chimeric structures and sulfurization
utilizing 3,4-dihydro-2H-benzothiole-3-one 1,1 dioxide (Beaucage
Reagent) to generate the phosphorothioate internucleotide
linkages for the center gap.

15 Other chimeric oligonucleotides, chimeric oligo-
nucleosides and mixed chimeric oligonucleotides/oligo-
nucleosides are synthesized according to United States
patent 5,623,065, herein incorporated by reference.

Example 6

20 **Oligonucleotide Isolation**

After cleavage from the controlled pore glass column
(Applied Biosystems) and deblocking in concentrated
ammonium hydroxide at 55°C for 18 hours, the
oligonucleotides or oligonucleosides are purified by
25 precipitation twice out of 0.5 M NaCl with 2.5 volumes
ethanol. Synthesized oligonucleotides were analyzed by
polyacrylamide gel electrophoresis on denaturing gels and
judged to be at least 85% full length material. The
relative amounts of phosphorothioate and phosphodiester
30 linkages obtained in synthesis were periodically checked by
³¹P nuclear magnetic resonance spectroscopy, and for some
studies oligonucleotides were purified by HPLC, as
described by Chiang et al., *J. Biol. Chem.* **1991**, 266,

18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

5 Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester
10 internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-
15 cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected
20 beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in
25 sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

30 The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual

products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing Electrospray-Mass Spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

20 T-24 cells:

The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

5 A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD)
10 supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached
15 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium
20 (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained
25 from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the
30 supplier.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of
5 OPTI-MEM™-1 containing 3.75 μ g/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours
10 after oligonucleotide treatment.

Example 10

Analysis of oligonucleotide inhibition of TRADD expression

Antisense modulation of TRADD expression can be assayed in a variety of ways known in the art. For
15 example, TRADD mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA.
20 Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et
25 al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems,
30 Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

TRADD protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting); ELISA or fluorescence-activated cell sorting (FACS). Antibodies
5 directed to TRADD can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for
10 example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp.
15 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western
20 blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and
25 can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

Poly(A)+ mRNA isolation

30 Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, **1996**, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular*

Biology, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90° hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and

attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

Example 13

Real-time Quantitative PCR Analysis of TRADD mRNA Levels

Quantitation of TRADD mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA

or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). TRADD probes and primers were designed to hybridize to the human TRADD sequence, using published sequence information

(GenBank accession number L41690, incorporated herein as SEQ ID NO:1).

For TRADD the PCR primers were:

forward primer: ACGAGGAGCGCTGTTTGAGT (SEQ ID No. 2)

5 reverse primer: TCCAGCTCAGCCAGTTCTTCAT (SEQ ID No. 3) and

the PCR probe was: FAM-CCAGCAGCCCGACCGGCTC-TAMRA

(SEQ ID No. 4) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

10 For GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID No. 5)

reverse primer: GAAGATGGTGATGGGATTTTC (SEQ ID No. 6) and the

PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID No. 7) where JOE (PE-Applied Biosystems, Foster City,

15 CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of TRADD mRNA levels

Eighteen hours after antisense treatment, cell
20 monolayers were washed twice with cold PBS and lysed in 1
mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA
was prepared following manufacturer's recommended
protocols. Twenty micrograms of total RNA was fractionated
by electrophoresis through 1.2% agarose gels containing
25 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.
Solon, OH). RNA was transferred from the gel to HYBOND™-N+
nylon membranes (Amersham Pharmacia Biotech, Piscataway,
NJ) by overnight capillary transfer using a
Northern/Southern Transfer buffer system (TEL-TEST "B"
30 Inc., Friendswood, TX). RNA transfer was confirmed by UV
visualization. Membranes were fixed by UV cross-linking
using a STRATALINKER™ UV Crosslinker 2400 (Stratagene,
Inc, La Jolla, CA).

Membranes were probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions with a TRADD specific probe prepared by PCR using the forward primer
5 ACGAGGAGCGCTGTTTGAGT (SEQ ID No. 2) and the reverse primer
TCCAGCTCAGCCAGTTCTTCAT (SEQ ID No. 3). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).
10 Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

15 Antisense inhibition of TRADD expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human TRADD RNA, using published sequences (GenBank
20 accession number L41690, incorporated herein as SEQ ID NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. L41690), to which the oligonucleotide binds. All compounds in Table
25 1 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on TRADD mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D."
30 indicates "no data".

Table 1
Inhibition of TRADD mRNA levels by phosphorothioate
oligodeoxynucleotides

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
5	19799	Coding	1	ggttcccacgcccgccag	0	8
	19800	Coding	31	ctcacctcctggccgcct	24	9
	19801	Coding	40	agctgccatctcacctcc	0	10
	19802	Coding	61	ctcttcgtgccattttg	11	11
	19803	Coding	67	caccactcttcgtgcc	0	12
10	19804	Coding	73	gctgcccacccactcttc	0	13
	19805	Coding	80	ggtatgcgctgcccaccc	0	14
	19806	Coding	90	tccacaaacaggtatgcg	0	15
	19807	Coding	96	gaggactccacaaacagg	7	16
	19808	Coding	103	gtccagcgaggactccac	64	17
15	19809	Coding	110	ccaccttgtccagcgagg	41	18
	19810	Coding	123	gcacccgacaggaccacc	7	19
	19811	Coding	129	gcgtaggcatccgacagg	3	20
	19812	Coding	133	gtgcgcgtaggcatccga	0	21
	19813	Coding	193	cccgccgctctctgccaa	0	22
20	19814	Coding	224	ggatcttcagcatctgca	0	23
	19815	Coding	230	tgcggtggatcttcagca	0	24
	19816	Coding	240	tgcgggtcgctgcggtgg	31	25
	19817	Coding	269	gcccgcagaatcgcagct	25	26
	19818	Coding	293	ggaggaagcggccacagg	32	27
25	19819	Coding	362	agtgctgggcgagcgcg	0	28
	19820	Coding	372	agcggcaccgagtgcctg	0	29
	19821	Coding	378	agttgcagcggcaccgag	0	30
	19822	Coding	384	agctccagttgcagcggc	18	31
	19823	Coding	420	gccagcaaagcgtccagc	0	32
30	19824	Coding	448	gatgcaactcaaacagcg	0	33
	19825	Coding	456	tgggctaggatgcaactc	33	34
	19826	Coding	461	gctgctgggctaggatgc	16	35
	19827	Coding	468	cggtcgggctgctgggct	0	36
	19828	Coding	492	tcagccagttcttcatcc	0	37
35	19829	Coding	510	cgcagcgcacccctccagc	1	38
	19830	Coding	516	agatttcgcagcgcaccc	0	39
	19831	Coding	526	gccgcacttcagatttcg	0	40
	19832	Coding	532	ccccgagccgcacttcag	23	41
	19833	Coding	563	ccgaagcgacctccccgt	0	42
40	19834	Coding	596	ccgacagagagggcaccg	0	43
	19835	Coding	617	gcggcgggcgggcggttca	0	44
	19836	Coding	624	ggtggcgggcgggcgggc	1	45
	19837	Coding	629	gggcaggtggcgggcgggc	30	46
	19838	Coding	636	aaagtctgggcaggtggc	44	47
45	19839	Coding	644	ggaacagaaaagtctggg	7	48
	19840	Coding	650	gaccctggaacagaaaag	10	49
	19841	Coding	662	tcactacaggctgaccct	26	50
	19842	Coding	666	cgattcactacaggctga	14	51

	19843	Coding	685	gtccttcagggtcagcg	25	52
	19844	Coding	692	tctgttggtccttcaggc	0	53
	19845	Coding	720	catttgagacccacagag	17	54
	19846	Coding	727	cttgcgccatttgagacc	30	55
5	19847	Coding	741	agtgagcgccccaccttg	2	56
	19848	Coding	763	cagcgcccggcagcctcg	0	57
	19849	Coding	783	gagtcagcgccgggtcc	52	58
	19850	Coding	794	cgtaggccagcgagtgcca	0	59
	19851	Coding	803	gctcgtactcgtaggcca	0	60
10	19852	Coding	810	ccctcgcgctcgtactcg	58	61
	19853	Coding	822	tgctcgtacagtcctcg	0	62
	19854	Coding	851	gcacgaagcgccgcagca	0	63
	19855	Coding	930	tcctctgccaggctgggtg	0	64
	19856	Coding	939	cccagcaagtcctctgcc	25	65
15	19857	Coding	964	caggccgcccattgggatc	0	66
	19858	Stop	986	tggctgcacccctgggtct	0	67
	19859	3' UTR	1004	tccaggttctccaaaagc	54	68
	19860	3' UTR	1016	accctaaggccatccagg	36	69
	19861	3' UTR	1032	aatagccgcagaagggaac	17	70
20	19862	3' UTR	1063	tcagggtcccgtggatgg	0	71
	19863	3' UTR	1079	taggccaaagtggagtttc	42	72
	19864	3' UTR	1093	gcagggtccagcagatagg	10	73
	19865	3' UTR	1117	gggaaggcaatcaactct	0	74
	19866	3' UTR	1161	gaggcagaatccccaatg	0	75
25	19867	3' UTR	1176	tctatcaaagtacctgag	0	76
	19868	3' UTR	1188	cccaccccacactctatc	53	77
	19869	3' UTR	1219	aagggtgaggctgatctcc	28	78
	19870	3' UTR	1228	ggatgggagaagggtgagg	45	79
	19871	3' UTR	1262	aaactgtaagggtggct	9	80
30	19872	3' UTR	1286	aaagatcaagggtgcttca	0	81
	19873	3' UTR	1303	atgaagtccaggacacca	0	82
	19874	3' UTR	1339	ctgttttacttcactgca	0	83
	19875	3' UTR	1351	caagattgattcctgttt	6	84
	19876	3' UTR	1378	ccacgctgagtggtgagct	32	85
35	19877	3' UTR	1409	actttattatcattgctt	34	86
	19878	3' UTR	1418	ccgtgttatactttatta	0	87

As shown in Table 1, SEQ ID NOs 9, 17, 18, 25, 26, 27, 34, 41, 46, 47, 50, 52, 55, 58, 61, 65, 68, 69, 72, 77, 78, 79, 85 and 86 demonstrated at least 20% inhibition of TRADD expression in this assay and are therefore preferred.

Example 16:**Antisense inhibition of TRADD expression- phosphorothioate 2'-MOE gapmer oligonucleotides**

In accordance with the present invention, a second series of oligonucleotides targeted to human TRADD were synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. L41690), to which the oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 2

Inhibition of TRADD mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
20039	Coding	1	ggttcccacgcccgccag	0	8
20040	Coding	31	ctcacctcctggcgcct	39	9
20041	Coding	40	agctgccatctcacctcc	51	10
30 20042	Coding	61	ctcttcgtgccattttg	53	11
20043	Coding	67	caccactcttcgtgccc	50	12
20044	Coding	73	gctgccaccactcttc	74	13
20045	Coding	80	ggtatgcgctgcccaccc	88	14
20046	Coding	90	tccacaaacaggtatgcg	67	15
35 20047	Coding	96	gaggactccacaaacagg	N.D.	16

	20048	Coding	103	gtccagcgcgaggactccac	70	17
	20049	Coding	110	ccaccttggtccagcgcagg	N.D.	18
	20050	Coding	123	gcacccgcagcaggaccacc	70	19
	20051	Coding	129	gcgtaggcatccgcagcagg	N.D.	20
5	20052	Coding	133	gtgcgcgtaggcacccga	79	21
	20053	Coding	193	cccgcgcgtctctgcgcaa	13	22
	20054	Coding	224	ggatcttcagcatctgca	N.D.	23
	20055	Coding	230	tgcgggtggatcttcagca	50	24
	20056	Coding	240	tgcgggtgcgctgcgggtgg	15	25
10	20057	Coding	269	gcccgcagaatcgcagct	43	26
	20058	Coding	293	ggaggaagcggccacagg	66	27
	20059	Coding	362	agtgcctgggcgagcgcgg	23	28
	20060	Coding	372	agcggcaccgagtgctgg	66	29
	20061	Coding	378	agttgcagcggcaccgag	24	30
15	20062	Coding	384	agctccagttgcagcggc	63	31
	20063	Coding	420	gccagcaaagcgtccagc	75	32
	20064	Coding	448	gatgcaactcaaacagcg	13	33
	20065	Coding	456	tgggctaggatgcaactc	44	34
	20066	Coding	461	gctgctgggctaggatgc	47	35
20	20067	Coding	468	cggtcgggctgctgggct	40	36
	20068	Coding	492	tcagccagttcttcatcc	0	37
	20069	Coding	510	cgcagcgcacatcctccagc	78	38
	20070	Coding	516	agatttcgcagcgcaccc	48	39
	20071	Coding	526	gccgcacttcagatttcg	38	40
25	20072	Coding	532	ccccgagccgcacttcag	10	41
	20073	Coding	563	ccgaagcgacctccccgt	1	42
	20074	Coding	596	ccgacagagagggcaccg	46	43
	20075	Coding	617	gcggcggcggcggcgttca	84	44
	20076	Coding	624	ggcggcggcggcggcggc	24	45
30	20077	Coding	629	gggcagggtggcggcggcg	11	46
	20078	Coding	636	aaagtctgggcagggtggc	47	47
	20079	Coding	644	ggaacagaaaagtctggg	16	48
	20080	Coding	650	gaccctggaacagaaaag	N.D.	49
	20081	Coding	662	tcactacaggctgaccct	46	50
35	20082	Coding	666	cgattcactacaggctga	17	51
	20083	Coding	685	gtccttcaggctcagcgg	69	52
	20084	Coding	692	tctgttggtccttcaggc	40	53
	20085	Coding	720	catttgagacccacagag	1	54
	20086	Coding	727	cttgcgccatttgagacc	40	55
40	20087	Coding	741	agtgagcgcgccaccttg	70	56
	20088	Coding	763	cagcgcgcggcagcctcg	38	57
	20089	Coding	783	gagtcagcgcgggtcc	56	58
	20090	Coding	794	cgtaggccagcaggtcca	43	59
	20091	Coding	803	gctcgtactcgtaggcca	82	60
45	20092	Coding	810	ccctcgcgctcgtactcg	76	61
	20093	Coding	822	tgctcgtacagtccttcg	62	62
	20094	Coding	851	gcacgaagcgcgcagca	0	63
	20095	Coding	930	tcctctgccaggctggtg	69	64
	20096	Coding	939	cccagcaagtcctctgcc	29	65
50	20097	Coding	964	caggccgccattgggatc	57	66
	20098	Stop	986	tggctgcacccctggtct	64	67
	20099	3' UTR	1004	tccagggttctccaaaagc	50	68

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	20100	3'	UTR	1016	accctaaggccatccagg	49	69
	20101	3'	UTR	1032	aatagccgcagaaggaac	0	70
	20102	3'	UTR	1063	tcaggggtcccgtggatgg	75	71
	20103	3'	UTR	1079	taggccaaagtggagtttc	60	72
5	20104	3'	UTR	1093	gcagggtccagcagatagg	79	73
	20105	3'	UTR	1117	gggaaggcaatcaactct	0	74
	20106	3'	UTR	1161	gaggcagaatccccaatg	60	75
	20107	3'	UTR	1176	tctatcaaagtacctgag	16	76
	20108	3'	UTR	1188	cccacccccacactctatc	0	77
10	20109	3'	UTR	1219	aagggtgaggctgatctcc	77	78
	20110	3'	UTR	1228	ggatgggagaagggtgagg	14	79
	20111	3'	UTR	1262	aaactgtaagggtggtct	67	80
	20112	3'	UTR	1286	aaagatcaagggtgcttca	50	81
	20113	3'	UTR	1303	atgaagtccaggacacca	53	82
15	20114	3'	UTR	1339	ctgttttacttcactgca	74	83
	20115	3'	UTR	1351	caagattgattcctgttt	68	84
	20116	3'	UTR	1378	ccacgctgagtgatgagct	81	85
	20117	3'	UTR	1409	actttattatcattgctt	72	86
	20118	3'	UTR	1418	ccgtgttatactttatta	0	87

20 As shown in Table 2, SEQ ID NOs 9, 10, 11, 12, 13, 14,
15, 17, 19, 21, 24, 26, 27, 29, 31, 32, 34, 35, 36, 38, 39,
40, 43, 44, 47, 50, 52, 53, 55, 56, 57, 58, 59, 60, 61, 62,
64, 66, 67, 68, 69, 71, 72, 73, 75, 78, 80, 81, 82, 83, 84,
85 and 86 demonstrated at least 30% inhibition of TRADD
25 expression in this experiment and are therefore preferred.

Example 17**Western blot analysis of TRADD protein levels**

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 hr after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to TRADD is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).